

# The Ubiquitin-Proteasome Pathway Is Required for Processing the NF- $\kappa$ B1 Precursor Protein and the Activation of NF- $\kappa$ B

Exhibit A

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## Summary

We demonstrate an essential role for the proteasome complex in two proteolytic processes required for activation of the transcription factor NF- $\kappa$ B. The p105 precursor of the p50 subunit of NF- $\kappa$ B is processed in vitro by an ATP-dependent process that requires proteasomes and ubiquitin conjugation. The C-terminal region of p105 is rapidly degraded, leaving the N-terminal p50 domain. p105 processing can be blocked in intact cells with inhibitors of the proteasome or in yeast with proteasome mutants. These inhibitors also block the activation of NF- $\kappa$ B and the rapid degradation of I $\kappa$ B $\alpha$  induced by tumor necrosis factor  $\alpha$ . Thus, the ubiquitin-proteasome pathway functions not only in the complete degradation of polypeptides, but also in the regulated processing of precursors into active proteins.

## Introduction

The transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) regulates a variety of genes involved in the immune and inflammatory responses. For example, NF- $\kappa$ B is required for the expression of the immunoglobulin light chain  $\kappa$  gene, the interleukin 2 receptor  $\alpha$  chain gene, the class I major histocompatibility complex (MHC) gene and a number of cytokine genes such as those encoding interleukin 2, interleukin 6, granulocyte colony-stimulating factor, and interferon- $\beta$  (IFN $\beta$ ) (reviewed by Baeuerle, 1991; Liou and Baltimore, 1993; Grilli et al., 1993). Similarly, NF- $\kappa$ B has been implicated in the expression of the cell adhesion genes that encode E-selectin, ICAM, and VCAM-1 (reviewed by Collins, 1993). Finally, a connection between the role of NF- $\kappa$ B in the immune response and the replication of the human immunodeficiency virus (HIV) was provided by the observation that NF- $\kappa$ B specifically binds to the HIV enhancer/promoter (Nabel and Baltimore, 1987).

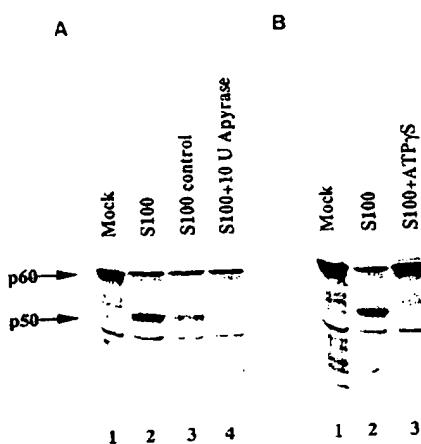
NF- $\kappa$ B was initially characterized as a heterodimeric complex consisting of a p50 (NF- $\kappa$ B1) and a p65 (RelA) subunit (Baeuerle and Baltimore, 1989; Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991; Ruben et al., 1991). The activity of NF- $\kappa$ B is highly regulated. In most cells, the p50 and p65 subunits of NF- $\kappa$ B form an inactive cytoplasmic ternary complex with the inhibitor protein I $\kappa$ B $\alpha$  (Baeuerle and Baltimore, 1988). I $\kappa$ B $\alpha$  masks the nuclear localization sequences of p50 and p65 (reviewed by Beg

and Baldwin, 1993). I $\kappa$ B $\alpha$  is rapidly degraded in response to a large number of extracellular signals, such as lipopolysaccharides, phorbol esters, and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and this step is obligatory for the activation of NF- $\kappa$ B (Beg et al., 1993; Brown et al., 1993; Henkel et al., 1993; Mellits et al., 1993; Sun et al., 1993). After degradation of I $\kappa$ B $\alpha$ , NF- $\kappa$ B translocates into the nucleus and activates gene expression.

The p50 subunit of NF- $\kappa$ B is generated by the proteolytic processing of a 105 kDa precursor protein (p105), and this processing is also regulated (Fan and Maniatis, 1991; Blank et al., 1991; Mellits et al., 1993; Mercurio et al., 1993). The C-terminus of p105 (p105C) contains ankyrin repeats and bears a striking resemblance to I $\kappa$ B $\alpha$  (Bours et al., 1990; Ghosh et al., 1990; Kieran et al., 1990). In fact, the lymphoid cell-specific I $\kappa$ B $\gamma$  protein is identical to p105C' (Inoue et al., 1992). However, this protein is generated by either alternative splicing or promoter usage. Significantly, unprocessed p105 can associate with p65 and other members of the Rel family to form inactive p105-p65 cytoplasmic complexes (Capobianco et al., 1992; Rice et al., 1992; Mercurio et al., 1993). Processing of p105 results in the production of p50, which can form the transcriptionally active p50-p65 heterodimer. The C-terminal I $\kappa$ B $\alpha$ -homologous sequence of p105 is rapidly degraded upon processing (Fan and Maniatis, 1991). Recent studies suggest that phosphorylation precedes cytokine-stimulated processing of p105 and degradation of I $\kappa$ B $\alpha$  (Beg et al., 1993; Brown et al., 1993; Mellits et al., 1993; Sun et al., 1994). However, neither the signal transduction pathways leading to NF- $\kappa$ B activation nor the mechanisms of I $\kappa$ B $\alpha$  inactivation or p105 processing are understood.

The p50 subunit of NF- $\kappa$ B is generated by processing of p105 in vivo and in vitro (Fan and Maniatis, 1991). The requirements of the in vitro processing reaction suggested that the ATP-dependent protease complex (e.g., proteasome), the ubiquitin-mediated (Ub-mediated) protein degradative pathway, or both are involved in p105 processing (Fan and Maniatis, 1991). The Ub-dependent pathway plays an important role in the complete degradation of abnormal and short-lived regulatory proteins (for reviews of the Ub-dependent pathway, see Goldberg, 1992; Hershko and Ciechanover, 1992; Rechsteiner et al., 1993). This pathway requires ATP and the covalent conjugation of target proteins with multiple Ub molecules (reviewed by Hershko and Ciechanover, 1992; Jentsch, 1992). In this multistep process, Ub is first activated by the enzyme E1, and the activated Ub is then transferred to one of a number of different Ub-conjugating enzymes (E2s) that catalyze the formation of an isopeptide bond between the C-terminal glycine of Ub and the  $\epsilon$ -amino group of lysine residues on target proteins. For many proteins, the conjugation to Ub also requires a specific Ub-protein ligase (E3). The monoubiquitinated substrate then undergoes further ubiquitinations via the lysine residue at position 48 on the Ubs, leading to the formation of multiUb chains (Chau et al., 1989) that target proteins for degrada-

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**Figure 1.** ATP Is Required for the Proteolytic Processing of the p105 Precursor In Vitro

The p105 precursor (p60Tth) was translated in a wheat germ extract, and the in vitro-translated [<sup>35</sup>S]-protein was mixed with HeLa cell cytoplasmic extract (S100) in processing buffer. After incubation at 30°C, the labeled proteins were immunoprecipitated with anti-p50 antibody, and the proteins were separated by SDS-PAGE.

(A) Apyrase inhibits the processing of p60Tth. Lane 1 is the substrate mock control incubated in the absence of HeLa extract. In lanes 2–4, p60Tth was incubated with HeLa cell S100. The p60 substrate was incubated without or with 10 U of apyrase at 30°C for 30 min (lanes 3 and 4, respectively) prior to the addition of HeLa cell S100. The reactions in lanes 3 and 4 did not receive additional ATP.

(B) ATPγS inhibits the formation of p50. The p60 precursor was incubated in the absence (lane 1) and presence of HeLa cell S100 (lanes 2 and 3). Reaction mixtures in lanes 1 and 2 contained 1 mM ATP. The processing reaction in lane 3 received 2 mM ATPγS.

tion by the 26S (1500 kDa) proteasome complex (Goldberg, 1992; Hershko and Ciechanover, 1992).

The 20S (600 kDa) proteasome particle (multicatalytic protease) comprises the catalytic core of the 26S particle (Eytan et al., 1989; Matthews et al., 1989; Driscoll and Goldberg, 1990). The proteasome is highly conserved and is present in the cytoplasm and nucleus of all eukaryotic cells. By itself, the 20S proteasome is unable to degrade ubiquitinated proteins, but through an ATP-dependent mechanism, it associates with other components to form the 26S proteasome complex capable of hydrolyzing Ub-conjugated proteins (Eytan et al., 1989; Driscoll and Goldberg, 1990).

Despite extensive studies in mammalian cells and yeast (Jentsch, 1992), thus far, only a few cellular targets of the Ub-dependent pathway have been identified. Recently, this pathway was shown to catalyze the generation of antigenic peptides from cytoplasmic proteins for presentation on MHC class I antigen molecules (Goldberg and Rock, 1992; Gaczynska et al., 1993; Michalek et al., 1993; Rock et al., 1994 [this issue of *Cell*]). We carried out experiments to determine whether the Ub–proteasome pathway is also involved in the ATP-dependent proteolytic processing of p105. We show here that the proteasome complex is required for p105 processing, and that this process requires Ub. In addition, we show that proteasome inhibitors block the rapid degradation of IκBα and the activation of NF-κB

upon TNFα induction. We conclude that the ATP-dependent proteasome complex plays a novel and essential role in the regulation of NF-κB activity.

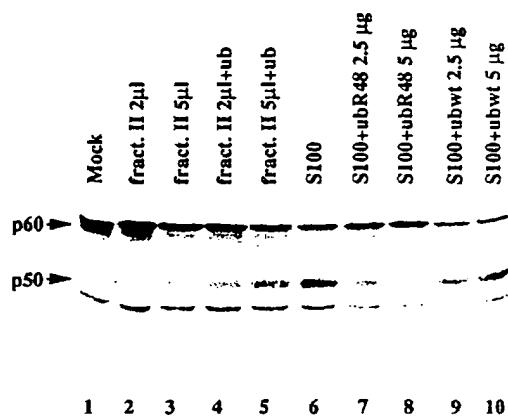
## Results

### ATP Is Required for p105 Processing

The p105 protein and the truncated version, p60Tth, can be produced by in vitro translation in a wheat germ extract and then processed to p50 in a HeLa cell cytoplasmic extract (S100) or in reticulocyte lysates (Fan and Maniatis, 1991). Both ATP and Mg<sup>2+</sup> were shown to be required for this reaction. We confirmed the requirement for ATP by showing that processing reactions treated with apyrase to destroy ATP are unable to generate p50 from p60Tth (Figure 1A). The p60Tth protein was used in these experiments because it is efficiently translated in vitro to generate the primarily full-length polypeptide. By contrast, p105 is inefficiently translated, and a large number of premature termination products, degradation products, or both are generated. Therefore, it is difficult to visualize the processing of p105 by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).

In vitro synthesized p60Tth mRNA was translated in a wheat germ extract to generate [<sup>35</sup>S]methionine-p60 protein. This protein was then incubated in the presence or absence of HeLa cell S100 extract and was immunoprecipitated with an anti-p50 antibody, and the immunoprecipitate was analyzed by SDS-PAGE. In the absence of the S100 extract, little if any p50 was observed (Figure 1A, lane 1). However, when the p60Tth translation reaction (which contains some ATP) was incubated with the S100 extract for 60 min, mature p50 was detected (Figure 1A, lane 3), and its production was stimulated by the addition of exogenous ATP (Figure 1A, lane 2). The same result was obtained when the reactions were immunoprecipitated with an anti-Myc peptide monoclonal antibody, which recognizes the Myc tag at the N-terminus of p60Tth (Fan and Maniatis, 1991). Thus, the p50 was generated by a proteolytic cleavage near the C-terminus of p60Tth. Similar results were also observed when full-length p105 was used in the reactions; however, the amount of processing was somewhat less than that with p60Tth (data not shown). Significantly, the formation of p50 was not observed if the in vitro translated p60Tth was first incubated with apyrase to remove any residual ATP left in the wheat germ extract (Figure 1A, lane 4). In addition, when the p60Tth precursor was incubated with the HeLa cell S100 extract in the presence of calf intestinal phosphatase, which also hydrolyzes ATP, the formation of p50 was not observed (data not shown).

To test whether ATP hydrolysis is necessary for processing, the reaction was carried out in the presence of the nonhydrolyzable analog ATPγS. As expected, processing of p60Tth in the HeLa cell S100 extract was observed in the presence of ATP (Figure 1B, lane 2). By contrast, no processing was observed in the presence of ATPγS (Figure 1B, lane 3). Although it is possible that the ATP is required for a phosphorylation event, we found that a panel of protein kinase inhibitors had no effect on



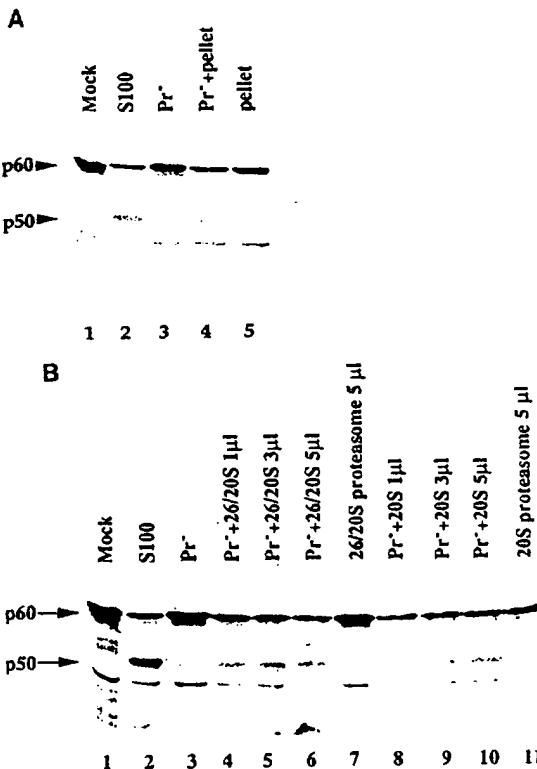
**Figure 2. Ub Is Required for the Processing of NF- $\kappa$ B1**  
Different amounts of reticulocyte fraction (fract.) II (in the presence [plus sign] and absence of 7.5  $\mu$ g of Ub) were used in an *in vitro* processing reaction with p60Tth as the substrate (lanes 2–5). In addition, HeLa cell S100, either alone (lane 6) or supplemented with two different amounts (2.5 or 5.0  $\mu$ g) of recombinant mutant UbR48 protein (lanes 7 and 8) or wild-type Ub protein (ubwt, lanes 9 and 10), was added to p60Tth in a processing reaction. Lane 1 is the control (Mock) incubated in the absence of either cytoplasmic extract.

processing (data not shown). Thus, it is likely that ATP hydrolysis is required for the processing reaction itself. Significantly, a series of ladder-like bands was observed above the unprocessed p60Tth band in the presence of ATP $\gamma$ S (Figure 1B, lane 3). This finding suggests Ub ligation to this protein since ATP $\gamma$ S supports the ubiquitination of proteins, but blocks proteasome-dependent degradation of ubiquitinylated proteins (Scheffner et al., 1992). Thus, by uncoupling these processes, ATP $\gamma$ S causes the accumulation of ubiquitinylated intermediates. These observations and the inhibition of processing by EDTA, hemin, and N-ethylmaleimide (Fan and Maniatis, 1991) strongly suggest that the Ub–proteasome pathway is required for p105 processing.

#### p105 Processing Requires Polyubiquitination

To test whether Ub is essential for p105 processing, we fractionated reticulocyte lysate to produce fraction II (Driscoll and Goldberg, 1990), which contains proteasomes and many Ub-conjugating enzymes but only low amounts of Ub. Incubation of the p60Tth protein with low amounts of fraction II and ATP led to the generation of low levels of p50 (Figure 2, lanes 2 and 3). These preparations of fraction II probably contain some Ub. Accordingly, the addition of Ub to fraction II significantly stimulated the formation of p50 (Figure 2, lanes 4 and 5).

The rapid degradation of proteins by the Ub pathway requires the formation of polyUb chains where Ub moieties are linked to Lys-48 on other Ub's (Chau et al., 1989; Hershko and Ciechanover, 1992; Jentsch, 1992). Mutant Ub proteins that contain an arginine at position 48 rather than a lysine do not form these Ub chains (Chau et al., 1989). The UbR48 mutant protein can therefore serve as a trans-dominant inhibitor of this degradation pathway. Thus, we compared the effects of wild-type Ub and the



**Figure 3. Proteasomes Stimulate the Processing of p60Tth In Vitro**  
(A) Processing of p60Tth in proteasome-depleted and -enriched extracts. HeLa cell S100 was centrifuged for 6 hr at 100,000  $\times$  g to remove proteasomes. The p60Tth precursor was incubated with buffer (Mock, lane 1), HeLa cell S100 (lanes 2), or proteasome-depleted HeLa cytoplasmic extract (Pr-, lanes 3). In lanes 4 and 5, the proteasome-enriched pellet from the ultracentrifugation step was added to the substrate in the presence and absence of Pr-, respectively.  
(B) Purified proteasomes stimulate the processing of p60Tth. Increasing amounts of purified 20S and 26S proteasomes (26/20S, lanes 4–6) or 20S proteasomes (lanes 8–10) from rabbit muscle were added in combination with p60Tth and Pr- extract in a processing reaction. In lanes 7 and 11, a mixture of 20S and 26S proteasomes and homogeneous 20S proteasomes, respectively, was added to p60Tth in the absence of any HeLa Pr- extract. Lane 1 is the mock-treated control, lane 2 is the HeLa S100-positive control, and lane 3 is the p60Tth processing reaction with the Pr- extract.

R48 variant on the processing of p60Tth (Figure 2, lanes 6–10). Both Ub forms were expressed in *Escherichia coli* with an N-terminal His-6 tag so that they could be easily purified using Ni<sup>2+</sup>-NTA chromatography (Beers and Callis, 1993). The addition of wild-type Ub to fraction II increased the formation of p50 (see above) and did not affect processing in HeLa S100 extract (Figure 2, lanes 9 and 10), presumably because Ub is not limiting in this extract. However, the addition of UbR48 to the HeLa S100 extract (Figure 2, lanes 7 and 8) or to reticulocyte fraction II (data not shown) significantly decreased the amount of p50 generated from p60Tth. Furthermore, in the presence of UbR48, a single band was observed migrating immediately above full-length p60Tth. This band is probably owing to the addition of a single UbR48 moiety to the substrate protein. Notably, the degradation of <sup>125</sup>I-lysozyme in reticu-

locyte fraction II or HeLa cytoplasmic extracts was inhibited by UbR48, while degradation of Ub-<sup>125</sup>I-lysozyme conjugates was not affected, indicating that UbR48 does not block processing by inhibiting proteasome activity but rather by blocking Ub conjugation (data not shown). We conclude that p60Tth is conjugated to multiple Ub chains in a fashion similar to the Ub conjugation of other substrates for the Ub-degradative pathway.

#### The Proteasome Is Required for p105 Processing

The multicatalytic 26S protease complex requires ATP and catalyzes the degradation of Ub-conjugated proteins (see above). Because generation of p50 requires ATP and the formation of polyUb chains, we examined whether p105 processing requires the 26S proteasome. Proteasomes were removed from the HeLa cell cytoplasmic S100 by differential centrifugation at 100,000 × g for 6 hr. The resulting proteasome-depleted supernatant (Pr<sup>-</sup>) contains a very low level of proteasome activity, while the pellet is enriched in proteasomes (Hegde et al., 1993). As expected, significant amounts of p50 were generated when p60Tth was incubated with the HeLa S100 extract (Figure 3A, lane 2), but removal of the proteasomes by centrifugation resulted in the loss of processing activity (Figure 3A, lane 3). Significantly, when the proteasome-rich pellet was added back to the supernatant, processing activity was restored (Figure 3A, lane 4). Interestingly, some p60Tth precursor was processed when incubated with the resuspended pellet alone (which contains some contaminating Ub-conjugating activity), but to a lesser extent than when incubated with the reconstituted extract (Figure 3A, compare lanes 4 and 5). Thus, the proteasomes are not sufficient for full processing activity, and other factor(s) in the Pr<sup>-</sup> extracts are required for maximal activity (see below). Additional evidence for the involvement of the proteasome in p105 processing was provided by the observation that the processing activity in the pellet was lost when the proteasomes were specifically immunodepleted from this fraction with an anti-human proteasome monoclonal antibody (data not shown).

After incubation of the p60 substrate in the proteasome-depleted extract (Figure 3A, lane 3), we observed ladder-like bands larger than full-length p60Tth and similar to those seen in Figure 1B (lane 3). When a large excess of Ub is added to the reaction with Pr<sup>-</sup>, the number and formation of these bands significantly increase (data not shown). Presumably these bands are ubiquitinated forms of the precursor protein that accumulate in the Pr<sup>-</sup> extract because of the lack of proteasome activity. In addition, a <sup>32</sup>P-glutathione S-transferase-Ub (<sup>32</sup>P-GST-Ub) fusion protein (Scheffner et al., 1993) is conjugated to p60Tth during a processing reaction with Pr<sup>-</sup> (data not shown). These results suggest that ubiquitination is a prerequisite for p105 processing and that the proteasome must remove these polyUb chains during the proteolytic processing event.

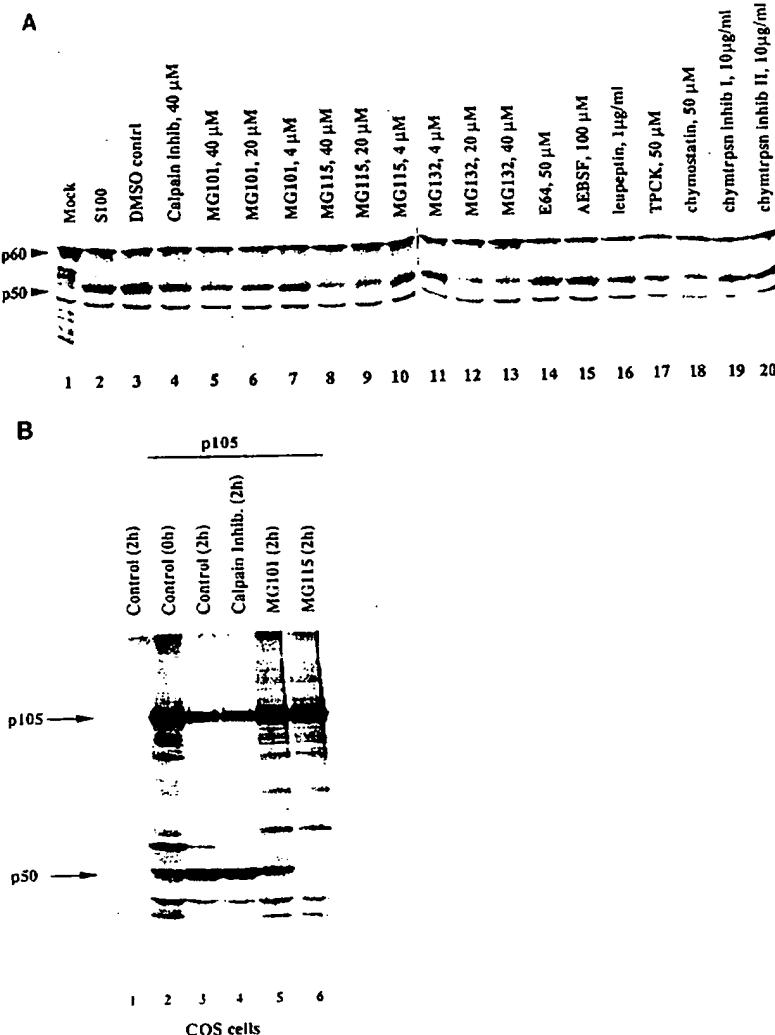
Additional evidence for the involvement of the proteasome in the processing of p60Tth was obtained by incubating p60Tth with increasing amounts of a purified mixture of 20S and 26S proteasomes (Figure 3B, lanes 4–6) or

pure 20S proteasomes (Figure 3B, lanes 8–10) in the Pr<sup>-</sup> extract. Both preparations were capable of restoring processing activity to the proteasome-depleted extract. In addition, a highly purified preparation of 26S proteasomes stimulated the formation of p50 when mixed with Pr<sup>-</sup> extract in a processing reaction (data not shown). However, p50 production was not observed when the precursor was incubated with purified proteasomes alone (Figure 3B, lanes 7 and 11). Thus, as described above for the pellet fraction (Figure 3A, lane 5), factor(s) in the Pr<sup>-</sup> extract are also required for the proteasomes to process p60Tth, presumably to allow Ub conjugation to the precursor proteins. It is interesting that processing was observed in the Pr<sup>-</sup> extract with 20S proteasomes, which by themselves cannot degrade Ub-conjugated proteins (Goldberg, 1992). However, the combination of 20S proteasomes with factors in the proteasome-depleted extract should allow formation of the 26S complex that acts on Ub-conjugated proteins. Accordingly, the addition of 20S proteasomes to the Pr<sup>-</sup> extract significantly enhanced the degradation of Ub-<sup>125</sup>I-lysozyme conjugates (data not shown).

#### Inhibitors of the Proteasome Block p105 Processing In Vitro and In Vivo

To obtain further evidence for the involvement of the proteasome in the formation of p50 in vitro and in vivo, we examined the effects of proteasome inhibitors on p105 processing. A series of peptide-aldehydes, MG101 (N-acetyl-leucinyl-leucinyl-norleucinal-H, also called LLnL), MG115 (carbobenzoxyl-leucinyl-leucinyl-norvalinal-H, also called Z-LLnV), and MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal-H, also called Z-LLL), have been shown to be potent inhibitors of the chymotryptic site on the 20S particle. These inhibitors have different dissociation constants (K<sub>d</sub>) against the 20S proteasome (MG101, 140 nM; MG115, 21 nM; MG132, 4.0 nM) (Rock et al., 1994). Previous studies have shown that MG101, which is also calpain inhibitor I, can inhibit the proteolytic activity of the proteasome (Visnitsky et al., 1992). MG101 and MG115 can also reduce the degradation of Ub-conjugated proteins in extracts (Rock et al., 1994). In addition, these inhibitors can block the degradation of long- and short-lived proteins in intact cells as well as the proteolytic generation of antigenic peptides presented on MHC class I molecules (Rock et al., 1994). We therefore analyzed whether these peptide-aldehyde inhibitors of the proteasome, as well as other protease inhibitors, can block the processing of p105.

Both MG115 and MG132 (at 20–40 μM) markedly inhibited the formation of p50 in HeLa S100 extracts (Figure 4A, lanes 8–13). By contrast, a less potent inhibitor of proteasome function, MG101 (Visnitsky et al., 1992; Rock et al., 1994), had only a very weak effect on the processing of p60Tth by HeLa S100 (Figure 4A, lanes 5–7). As revealed by quantitating the data on a PhosphorImager, none of the other protease inhibitors tested significantly affected p105 processing. These other inhibitors include calpain inhibitor II (Figure 4A, lane 4), E64 (lane 14), 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF, lane 15), leupeptin (lane 16), Nα-p-tosyl-L-phenylalanine chloro-



**Figure 4.** Inhibitors of the Proteasome Block the Formation of p50 In Vitro and In Vivo

(A) In vitro analysis. Lane 1 is the p60Tth mock control incubated without HeLa extract. HeLa cell S100 was added to p60Tth in the absence (lane 2) or presence of different inhibitors (lanes 4–20) in a processing reaction. The type and amount of inhibitor are listed above each lane. Lane 3 is the DMSO (0.4% final) buffer control.

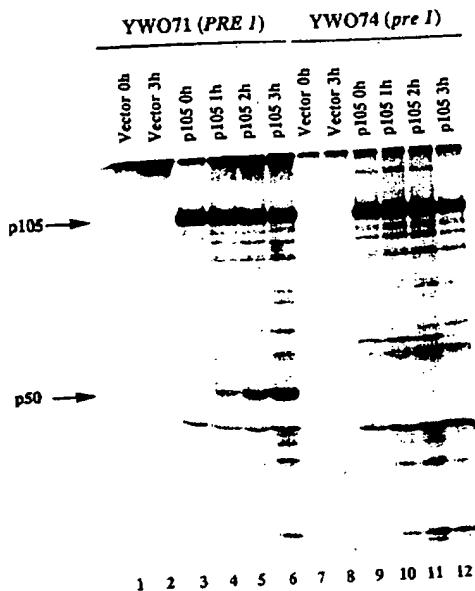
Abbreviations: contrl, control; inhib, inhibitor; and chymtrpsn, chymotrypsin.

(B) In vivo analysis. COS cells were transfected with vector alone (Control, lane 1) or with a human p105 expression construct (lanes 2–6). After transfection (48 hr), calpain inhibitor II (lane 4), MG101 (lane 5), and MG115 (lane 6) were added to the cells for 1 hr at a concentration of 50  $\mu$ M. The cells were then pulse-chased with [ $^{35}$ S]methionine-[ $^{35}$ S]cysteine, and the cell proteins were immunoprecipitated with anti-p50 antibody and analyzed by SDS-PAGE. Inhibitors were present throughout the entire pulse–chase period. Lane 2 is the 20 min pulse, 0 hr (0h) chase p105 control. In the vector control (lane 1) and the p105 controls (lanes 2 and 3), the cells were pretreated with 0.25% DMSO, the inhibitor diluent. The radio-labeled band present in lane 2 that is slightly larger than p50 could be either a short-lived intermediate of the processing reaction or it could be nonspecific since, in a subsequent experiment, anti-p50 antibody failed to immunoprecipitate this protein.

methyl ketone (TPCK, lane 17), chymostatin (lane 18), and chymotrypsin inhibitors I (lane 19) and II (lane 20). In contrast with the reports on intact cells by Henkel et al. (1993) and Mellits et al. (1993), TPCK in our experiments had no effect on processing in vitro. Surprisingly, ubiquitinated forms of p60Tth were not seen when the proteolytic activity of the proteasome was blocked with the inhibitors MG115 and MG132. This may be a consequence of the isopeptidase activity associated with the 26S proteasome, which hydrolyzes multiUb chains. This activity has been shown to be unaffected by these inhibitors (D. Hwang and A. L. G., unpublished data).

To determine whether these inhibitors can block p105 processing in vivo, we transfected monkey COS cells with a human p105 expression plasmid, and 48 hr after transfection, we treated the cells with various inhibitors for 1 hr or with dimethyl sulfoxide (DMSO) as a control. The cells were then pulse labeled with [ $^{35}$ S]methionine-[ $^{35}$ S]cysteine for 20 min, followed by a 2 hr chase period. Cell lysates were immunoprecipitated with anti-p50 antibody, and the proteins were separated by SDS-PAGE. When the cells were examined after the pulse label, the

p105 precursor was easily detected, but only low levels of radiolabeled p50 were present (Figure 4B, lane 2). After the chase period, the level of p105 decreased, but a significant amount of p50 was now detected (Figure 4B, lane 3), as reported previously (Fan and Maniatis, 1991). The addition of calpain inhibitor II (N-acetyl-leucinyl-leucinyl-methionyl-H, also called LLM) to the cells had no effect on the processing of p105 (Figure 4B, lane 4). This inhibitor is also a peptide-aldehyde, but it has only a slight effect on proteasome function (Rock et al., 1994) and on p105 processing in vitro (Figure 4A, lane 4). However, the proteasome inhibitors MG101 and MG115 dramatically reduced the amount of p50 (Figure 4B, lanes 5 and 6, respectively). MG115 at 50  $\mu$ M completely blocked the processing of p105, while MG101 inhibited it by approximately 70%. Surprisingly, MG101 had a greater inhibitory effect on p105 processing in intact cells than in extracts (compare Figure 4B, lane 5 with Figure 4A, lane 5, respectively), as was also observed for overall protein breakdown and for antigen presentation (Rock et al., 1994). In a similar experiment, we examined the effects on p105 processing of the very potent proteasome inhibitor MG132 and the cyste-



**Figure 5.** Processing of p105 in *S. cerevisiae* Requires the Proteasome

Both wild-type YWO71 (*PRE1*) (lanes 1–6) and proteasome mutant YWO74 (*pre1*) (lanes 7–12) yeast were transformed with vector alone (lanes 1, 2, 7, and 8) or with a human p105 yeast expression vector (lanes 3–6 and 9–12). The transformants were pulsed with [<sup>35</sup>S]methionine–[<sup>35</sup>S]cysteine for 20 min and were chased with complete medium for 0 hr (0h, lanes 1, 3, 7, and 9), 1 hr (1h, lanes 4 and 10), 2 hr (2h, lanes 5 and 11), and 3 hr (3h, lanes 2, 6, 8, and 12). Cell lysates were immunoprecipitated with anti-p50 antibody, and the proteins were separated by SDS-PAGE.

ine protease inhibitor E64 (L-transepoxysuccinic acid). MG132 (5 μM) completely blocked the formation of p50 during the 2 hr chase period (data not shown), while E64 (50 μM) had no effect.

The relative order of efficacy of MG101, MG115, and MG132 on p105 processing agrees with their relative abilities to inhibit the hydrolysis of hydrophobic peptides, casein, and ubiquitinated proteins with the 26S proteasome (Rock et al., 1994; R. Stein, unpublished data). Although these aldehydes also inhibit calpain and lysosomal cathepsin, the three have similar *K<sub>s</sub>* (5–12 nM) against these cysteine proteases, which is in contrast with their very different effects on proteasomes (Rock et al., 1994) and p105 processing. Moreover, the lysosomal enzymes and calpains are inactivated by E64 and calpain inhibitor II, both of which had no effect on p105 processing. Consequently, these peptide-aldehydes must be blocking p105 processing by inhibiting proteasome function.

#### p105 Processing in Yeast Requires the Proteasome

The proteasome is highly conserved in all eukaryotes (Tanaka et al., 1992), and a number of mutants in the proteasome and the Ub pathway have been identified and characterized in *Saccharomyces cerevisiae*. We tested whether human p105 protein can also be processed in yeast, and if so, whether the proteasome is required for this activity. Therefore, the p105 cDNA was inserted into

a yeast expression vector, and the recombinant plasmid was introduced into a wild-type yeast strain (YWO71 strain; Seufert and Jentsch, 1992). A pulse-chase analysis in the wild-type strain revealed a precursor-product relationship between p105 and a protein of approximately 50 kDa (Figure 5, lanes 1–6). DNA binding studies with extracts from these cells indicated that the p50 protein binds specifically to NF-κB sites. In addition, the processed protein can stimulate the transcription of a reporter gene containing four NF-κB-binding sites (data not shown). Thus, the p50 produced in yeast is functional. A careful comparison of the electrophoretic mobility of the p50 generated in yeast revealed that it is approximately 2 kDa smaller than that produced in mammalian cells. The somewhat smaller size of the yeast p50 protein could be due to differences in the properties of the yeast degradative system. Alternatively, the structure of p105 produced in yeast may differ from that produced in mammalian cells, and this difference could expose an alternative proteolytic cleavage site. This difference in size could also be due to a posttranslational modification in mammalian cells that is absent in yeast.

Yeast carrying a missense mutation in the *PRE1* proteasome subunit gene, which inhibits the chymotrypsin-like activity of the proteasome, are defective in the rapid degradation of abnormal and short-lived proteins (Heinemeyer et al., 1991; Seufert and Jentsch, 1992). The degradation of such polypeptides is achieved via the Ub-dependent pathway and requires the Ub-conjugating enzymes (E2s), UBC4 and UBC5 (Seufert and Jentsch, 1990). When a similar pulse-chase experiment was carried out in the *pre1* mutant (YWO74 strain; Seufert and Jentsch, 1992), the processed p50 was barely detectable (Figure 5, lanes 7–12). Furthermore, when the steady-state levels of p105 and p50 were measured in these strains by Western blotting analysis, very little p50 was detected in the *pre1* mutant yeast compared with the levels in the wild-type *PRE1* strain (data not shown). However, the levels of p105 in these strains were comparable. Similarly, the amounts of p50-specific DNA binding and transcriptional activity were dramatically decreased in the mutant *pre1* strain compared with that in the wild-type strain. We conclude that the proteasome is required for p105 processing in yeast, as in mammalian cells.

#### Proteasome Inhibitors Block the Inducible Degradation of IκBα in Mammalian Cells

An important feature of p105 processing in mammalian cells (Fan and Maniatis, 1991), *in vitro* and in yeast (V. J. P. and T. M., unpublished data), is that the C-terminal half of p105 (p105C') is rapidly degraded during the production of p50. The striking similarities between the structure, function, and instability of p105C' and IκBα suggest that similar mechanisms may be involved in their degradation. Therefore, we determined whether the proteasome is also involved in the rapid degradation of IκBα.

Previous studies have shown that TNFα induces the rapid degradation of IκBα and the activation of NF-κB (Beg et al., 1993; Brown et al., 1993; Henkel et al., 1993; Mellits et al., 1993; Sun et al., 1993, 1994). To determine whether

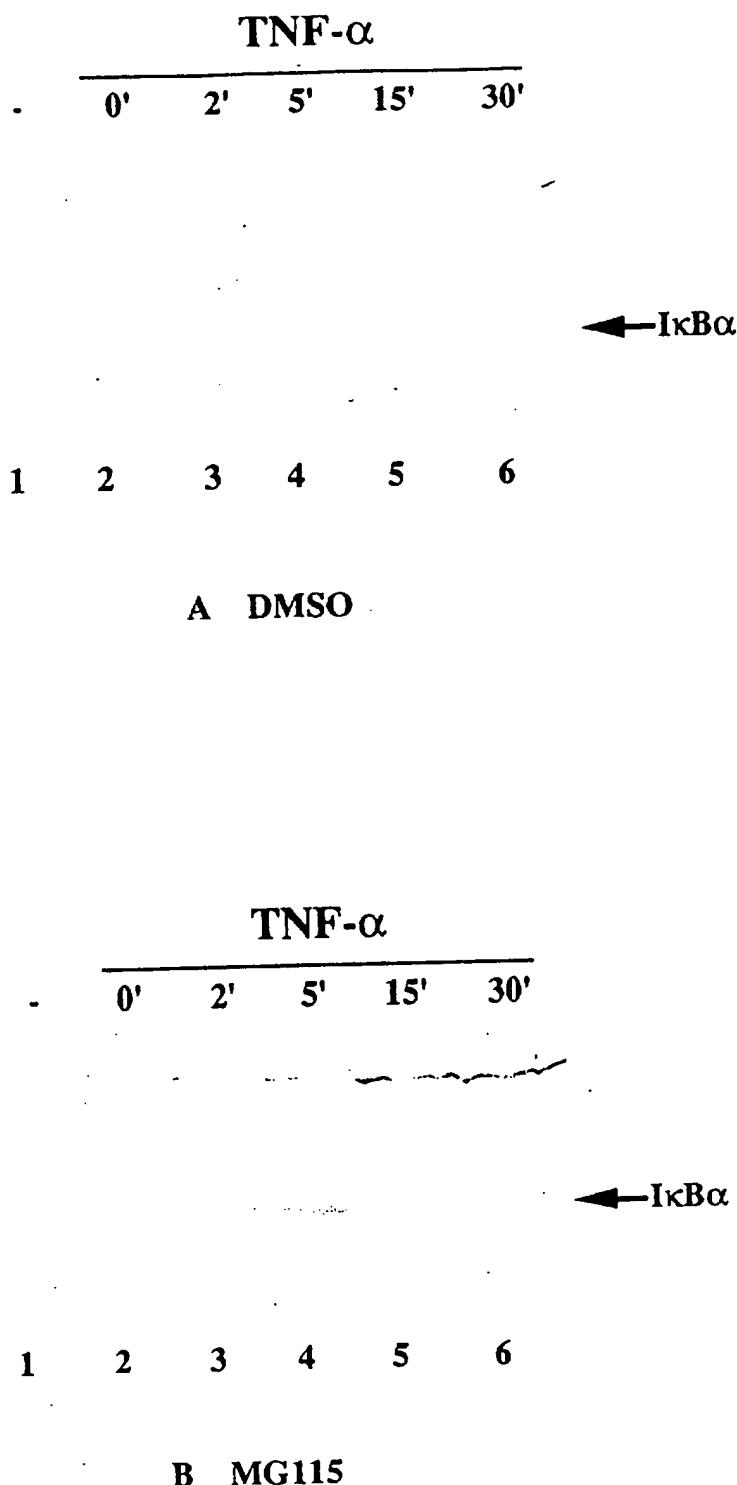
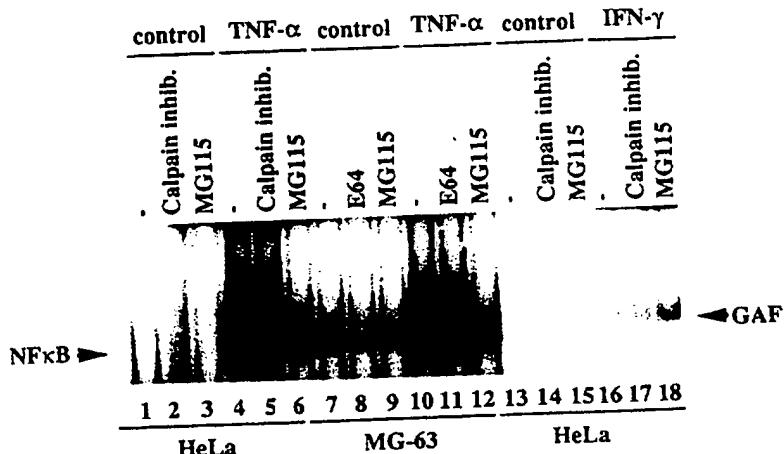


Figure 6. Proteasome Inhibitors Block TNF- $\alpha$ -Stimulated Degradation of I $\kappa$ B $\alpha$

Human MG-63 cells were pretreated with DMSO (0.125%) (A) or the proteasome inhibitor MG115 (50  $\mu$ M) (B) for 1 hr. The cells were then treated with TNF- $\alpha$  (1000 U/ml) for 0 min (lanes 1 and 2), 2 min (lane 3), 5 min (lane 4), 15 min (lane 5), and 30 min (lane 6). In lane 1, the cells did not receive DMSO (A) or MG115 (B). The degradation of I $\kappa$ B $\alpha$  was followed by Western blot analysis of whole-cell lysates using an I $\kappa$ B $\alpha$ -specific antiserum. I $\kappa$ B $\alpha$  is indicated by an arrow, and the phosphorylated form of I $\kappa$ B $\alpha$  (upper band of doublet) can be seen in (B), lanes 5 and 6.

proteasome inhibitors can prevent the degradation of I $\kappa$ B $\alpha$ , we preincubated human MG-63 cells with or without the inhibitors MG115 (Figure 6) or MG132 (data not shown) for 1 hr prior to TNF- $\alpha$  treatment. The level of I $\kappa$ B $\alpha$  was then followed as a function of time by Western blot analysis using an I $\kappa$ B $\alpha$ -specific antiserum (Beg et al., 1993). As previously described, I $\kappa$ B $\alpha$  is rapidly degraded within 15 min after the addition of TNF- $\alpha$  (Figure 6A, lanes 1–6). In

striking contrast, the proteasome inhibitors MG115 (Figure 6B, lanes 1–6) and MG132 (data not shown) completely blocked the degradation of I $\kappa$ B $\alpha$  in response to TNF- $\alpha$ . The cysteine protease inhibitors E64 and calpain inhibitor II had no effect on the degradation of I $\kappa$ B $\alpha$  (data not shown). Significantly, MG132 (data not shown) and MG115 appeared to stabilize the phosphorylated form of I $\kappa$ B $\alpha$  that appeared in response to TNF (note the appearance of a



**Figure 7.** Inhibitors of the Proteasome Block TNF $\alpha$ -Stimulated Activation of NF- $\kappa$ B

HeLa (lanes 1–6 and 13–18) or MG-63 (lanes 7–12) cells were pretreated with medium (lanes 7 and 10), 0.125% DMSO (lanes 1, 4, 13, and 16), 50  $\mu$ M calpain inhibitor (inhib.) II (lanes 2, 5, 14, and 17), 50  $\mu$ M E64 (lanes 8 and 11), or 50  $\mu$ M MG115 (lanes 3, 6, 9, 12, 15, and 18) for 1 hr. Cells were then treated with TNF $\alpha$  (1000 U/ml) (lanes 4–6 and 10–12) or IFN $\gamma$  (1000 U/ml) (lanes 16–18) for 30 and 60 min, respectively. Whole-cell extracts were prepared and analyzed by electrophoretic mobility shift assay. The PRDII probe containing the NF- $\kappa$ B-binding sites from the human IFNB gene was used to measure NF- $\kappa$ B binding activity, and a –127 to –109 probe from the IFN regulatory factor 1 gene was used to measure GAF DNA binding activity.

doublet in Figure 6B at 15 and 30 min) (Beg et al., 1993). Treatment of these extracts with calf intestinal phosphatase converted the upper band of the doublet to the lower band (data not shown). Consistent with earlier observations (Beg et al., 1993; Brown et al., 1993; Mellits et al., 1993; Sun et al., 1994), this result suggests that I $\kappa$ B $\alpha$  is phosphorylated prior to its degradation. We conclude that the proteasome is involved in p105 processing and possibly in TNF $\alpha$ -induced degradation of I $\kappa$ B $\alpha$ .

#### Proteasome Inhibitors Block NF- $\kappa$ B Activation in Mammalian Cells

The results presented thus far indicate that the proteasome is required for both mechanisms of NF- $\kappa$ B activation: the processing of p105, which generates the active p50–p65 heterodimer of NF- $\kappa$ B, and the complete degradation of I $\kappa$ B $\alpha$ , which is necessary for the translocation of NF- $\kappa$ B into the nucleus, where it stimulates gene expression (Beg and Baldwin, 1993; Grilli et al., 1993). To directly demonstrate that the proteasome is required for NF- $\kappa$ B activation, we measured the effects of the peptide-aldehyde inhibitors of the proteasome on NF- $\kappa$ B activation using a DNA binding assay. HeLa and MG-63 cells were pretreated with these different inhibitors for 1 hr prior to treatment with TNF $\alpha$  or IFN $\gamma$ . NF- $\kappa$ B and  $\gamma$ -activated factor (GAF) (Sims et al., 1993) DNA-binding activities were measured in whole-cell extracts using an electrophoretic mobility shift assay. Very little NF- $\kappa$ B or GAF DNA-binding activities were present in the extracts from uninduced cells (Figure 7, lanes 1–3, 7–9, 13–15). Treatment of cells with TNF $\alpha$  (Figure 7, lanes 4–6, 10–12) resulted in a large stimulation of NF- $\kappa$ B binding activity. This activation was unaffected by the cysteine protease inhibitors calpain inhibitor II and E64 (Figure 7, lanes 5 and 11, respectively). By contrast, the proteasome inhibitors MG115 (Figure 7, lanes 6 and 12) and MG132 (data not shown) almost completely blocked the activation of NF- $\kappa$ B by TNF $\alpha$  in HeLa and MG-63 cells. In addition, we found that the activation of NF- $\kappa$ B by double-stranded RNA was also blocked by proteasome inhibitors (data not shown). Significantly, none of the inhibitors tested had any

effect on GAF induction by IFN $\gamma$  (Figure 7, lanes 16–18). We conclude that inhibitors of proteasome function specifically block NF- $\kappa$ B induction.

#### Discussion

##### Mechanisms of NF- $\kappa$ B Activation

We have shown that the proteasome plays an essential role in two distinct mechanisms for the activation of NF- $\kappa$ B (Figure 8). In the first mechanism, an inactive ternary complex consisting of NF- $\kappa$ B heterodimers and I $\kappa$ B $\alpha$  is activated by the inducible degradation of I $\kappa$ B $\alpha$ . Our results show that the rapid postinduction degradation of I $\kappa$ B $\alpha$  is blocked in vivo by proteasome inhibitors. In the second mechanism, the inactive heterodimer consisting of p105 and p65 is activated by the proteolytic processing of p105 to generate the active p50–p65 heterodimer. The I $\kappa$ B-like C-terminal region of p105 is rapidly degraded during this processing reaction. We have shown that, in vitro, the generation of p50 requires the proteasome and the formation of polyUb chains. In addition, we have shown that p105 processing in vitro and in vivo is blocked by proteasome inhibitors. As shown in the model of Figure 8, these proteasome inhibitors can block both pathways of NF- $\kappa$ B activation. Thus, it may be possible to completely block the physiological effects of a number of cytokines, since the activation of NF- $\kappa$ B is a common step in many different cytokine-dependent signal transduction pathways.

The two pathways of NF- $\kappa$ B activation illustrated in the model of Figure 8 could be regulated by the phosphorylation of I $\kappa$ B $\alpha$  and p105, by the activation of specific Ub-conjugating enzymes, by the activation of the proteasome, or by a combination of these mechanisms. Evidence for the first possibility is provided by the observation that both I $\kappa$ B $\alpha$  and p105 are rapidly phosphorylated in response to inducers of NF- $\kappa$ B in vivo and that NF- $\kappa$ B can be activated by phosphorylation in vitro (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990; Beg et al., 1993; Brown et al., 1993; Mellits et al., 1993; Sun et al., 1994). Although a number of studies suggest a role for phosphorylation in the activation of NF- $\kappa$ B (Neumann et al., 1992; Frantz et

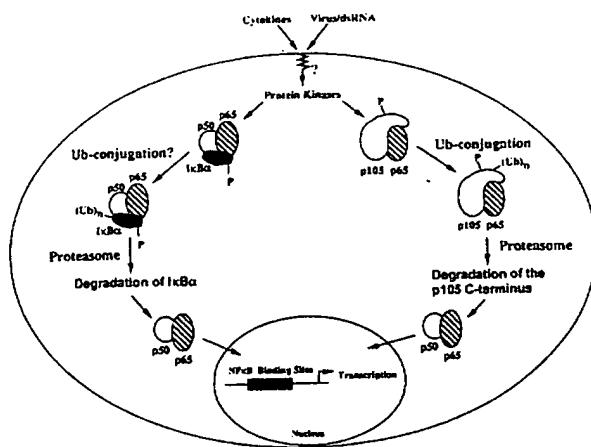


Figure 8. Role of the Proteasome in Two Different Pathways of NF- $\kappa$ B Activation

The transcriptional activity of heterodimers consisting of p50 and one of many Rel family proteins, such as p65, can be regulated by at least two mechanisms. First, the heterodimers associate with I $\kappa$ B $\alpha$  to form an inactive ternary complex. Second, p65 associates with p105 to form inactive complexes. Both complexes remain localized in the cytoplasm. The model shown here for NF- $\kappa$ B activation is consistent with all the information available, but some aspects of the model have not yet been directly tested. According to this model, a variety of extracellular signals or a viral infection activates signal transduction pathway(s) that lead to the phosphorylation of I $\kappa$ B $\alpha$  and p105. This modification may provide a recognition signal for ubiquitination. We have demonstrated that ubiquitination of p105 is required for the subsequent steps in the processing pathway, but we have not shown that ubiquitination of I $\kappa$ B $\alpha$  is a prerequisite for its degradation. In the next step of the model, the C-terminus of the ubiquitinated p105 is degraded to generate the p50 subunit of the p50-p65 heterodimer. In parallel, I $\kappa$ B $\alpha$  is degraded, which results in the appearance of functional p50-p65 heterodimers. We have shown that the proteasome is required for p105 processing and that proteasome inhibitors block the degradation of I $\kappa$ B $\alpha$ . Whether the proteasome degrades I $\kappa$ B $\alpha$  as it is bound to NF- $\kappa$ B or whether it does so after it dissociates remains to be established.

al., 1994), there is no direct evidence that phosphorylation is required for p105 processing or for the degradation of I $\kappa$ B $\alpha$  (Henkel et al., 1993).

In this paper, we show that the ubiquitination of p105 and possibly I $\kappa$ B $\alpha$  are required for NF- $\kappa$ B activation. We do not know whether phosphorylation of either I $\kappa$ B $\alpha$  or p105 precedes ubiquitination and, if so, whether phosphorylation in each case is required for this process. The latter possibility is suggested by the observation that phosphorylation triggers the Ub-dependent degradation of cyclins (D. Finley, personal communication). An alternative possibility is that phosphorylation leads to the dissociation of I $\kappa$ B $\alpha$  and p105 from their respective complexes (Henkel et al., 1993), and this results in their ubiquitination and degradation by the proteasome. However, we have shown that proteasome inhibitors stabilize a phosphorylated form of I $\kappa$ B $\alpha$ , and they completely block the activation of NF- $\kappa$ B. If phosphorylation of I $\kappa$ B $\alpha$  was sufficient for its dissociation from the ternary complex, NF- $\kappa$ B should be activated in the presence of proteasome inhibitors. This observation suggests that the Ub-proteasome pathway

may act on the bound proteins rather than on free I $\kappa$ B $\alpha$  or p105.

All of the mechanisms discussed above are based on the hypothesis that the initial step in NF- $\kappa$ B activation is the phosphorylation of I $\kappa$ B $\alpha$  and p105. An alternative possibility is that the initial step is the activation of specific Ub-conjugating enzymes or the alteration of proteasome activity. The former possibility is suggested by the observation that a specific Ub-conjugating enzyme is involved in human papilloma virus-mediated p53 degradation (Scheffner et al., 1993). An example of the latter possibility is the alteration of the proteolytic activity of the proteasome for antigen processing (Gaczynska et al., 1993; Boes et al., 1994).

#### A Novel Mechanism for Proteolytic Processing

There are many examples of protein processing in which a specific protease cleaves at a short recognition sequence to generate discrete peptide cleavage products (Barr, 1991). By contrast, the processing of p105 involves the complete degradation of the C-terminal 55 kDa of p105 to generate a stable 50 kDa N-terminal fragment (p50) (Fan and Maniatis, 1991; V. J. P. and T. M., unpublished data). Many studies have implicated the actions of proteasome and Ub in the complete degradation of proteins, but not in limited proteolysis, which yields active forms of proteins. A possible mechanism for the selective degradation of the C-terminus of p105 is the selective ubiquitination of sequences on the C-terminal half of p105. Proteolysis would then leave an unubiquitinated N-terminus and would result in the complete digestion of the ubiquitinated C-terminus. Both I $\kappa$ B $\alpha$  and the C-terminus of p105 contain PEST-like sequences (Mellits et al., 1993), an amino acid sequence motif believed to target proteins for degradation (Rogers et al., 1986). In fact, PEST sequences have recently been shown to participate in Ub-dependent protein breakdown (D. Finley, personal communication).

We note that the C-terminus of p105 is not only generated via a processing reaction, but it can also be generated by a separate mRNA in lymphoid cells (Inoue et al., 1992; Liou et al., 1992). This latter protein (I $\kappa$ B $\gamma$ /p70) is slightly larger than the expected C-terminal proteolytic cleavage product, and this size difference could be one reason for its greater stability. Alternatively, I $\kappa$ B $\gamma$  could also be stabilized through an interaction with p50 or p52. Another possible explanation for the differences in stability between the C-terminus of p105 and I $\kappa$ B $\gamma$  is that after proteolytic cleavage, the C-terminal fragment of p105 may have a destabilizing N-terminal amino acid and is, therefore, rapidly degraded via the Ub pathway by the N-end rule (reviewed by Varshavsky, 1992). However, it does not appear likely that this mechanism alone is responsible for the rapid degradation of p105C', since full-length p105 is ubiquitinated prior to undergoing proteolytic cleavage.

Taken together these observations suggest that p105 processing may not involve proteolytic cleavage at a specific site, followed by degradation of the C-terminus. Rather, the entire molecule may enter the normal Ub-proteasome degradative pathway, but the p50 terminal fragment may be protected from digestion either because

of some inherent structural feature or by an association with some inhibitory factor.

Recently, the protease inhibitors TPCK and Na-p-tosyl-L-lysine chloromethyl ketone (TLCK) were reported to inhibit the processing of p105 and the degradation of I $\kappa$ B $\alpha$  and to block the activation of NF- $\kappa$ B (Henkel et al., 1993; Mellits et al., 1993). These compounds, however, are quite reactive as alkylating agents, and they have little or no effect on proteasome activity (Hough et al., 1987). Therefore, they may affect p105 processing by either an indirect or nonspecific mechanism. In fact, we found that TPCK has no effect on the formation of p50 in vitro (Figure 4A). In addition, recent studies have shown that TPCK and TLCK block the phosphorylation of I $\kappa$ B $\alpha$  (Mellits et al., 1993; T. Finco and A. Baldwin, personal communication). By contrast, the proteasome inhibitors block the processing of p105 (Figures 4A and 4B) and the degradation of I $\kappa$ B $\alpha$ , but do not appear to block the phosphorylation of I $\kappa$ B $\alpha$  (Figure 6). Therefore, the effects of the protease inhibitors TPCK and TLCK on NF- $\kappa$ B activation appear to act upstream from the proteolysis of p105 and I $\kappa$ B $\alpha$ .

We cannot eliminate the possibility that MG115 and MG132 inhibit p105 processing and I $\kappa$ B $\alpha$  degradation by affecting some unidentified cell enzyme. However, our findings in cell-free extracts of a requirement for substrate conjugation to multiple Ub's, for the proteasome and for ATP hydrolysis, and the failure of p105 processing in vivo in yeast proteasome mutants indicate that the effect of these inhibitors is on the 26S proteasome. Furthermore, the present findings are in agreement with the extensive biochemical data of Rock et al. (1994) indicating that these peptide-aldehydes block the breakdown of proteins by the Ub-proteasome pathway in vivo.

Proteolytic processing events may also be required in signal transduction pathways leading to the activation of the transcription factor *dorsal* in Drosophila. The *dorsal* protein, which is a member of the NF- $\kappa$ B/Rel family of proteins (Grilli et al., 1993), establishes the dorsal-ventral pattern of the Drosophila embryo. Similar to the NF- $\kappa$ B-I $\kappa$ B $\alpha$  interaction, the *dorsal* protein is complexed to *cactus*, an I $\kappa$ B homolog (Geisler et al., 1992; Kidd, 1992). Significantly, the *pelle* gene, which encodes a protein kinase, is required for nuclear import of the *dorsal* protein (Shelton and Wasserman, 1993). It is possible that this kinase phosphorylates *cactus*, *dorsal*, or both, resulting in the rapid degradation of *cactus* (Whalen and Steward, 1993). Because of the striking similarities in the activation of NF- $\kappa$ B and *dorsal* and because of the conservation of components of the Ub-proteasome pathway, it seems likely that the degradation of *cactus* in the ventral region of the Drosophila embryo requires the proteasome.

In mammalian cells, the HIV1 protease has been shown to process the p105 precursor protein to a p45 rather than to a p50 protein, both in vitro and in vivo (Riviere et al., 1991). Thus, the products of HIV1 protease cleavage and proteasome-dependent processing are distinct. In intact cells, the processing of p105 is a highly regulated event. Therefore, the generation of p50 by the HIV1 protease may work independently of the proteasome. This protease may be required to increase the amount of NF- $\kappa$ B in HIV1-

infected cells and thus increase the level of viral gene expression (Riviere et al., 1991).

In addition to NF- $\kappa$ B, a number of eukaryotic transcription factors are regulated by proteolytic processing. For example, the two subunits of the general transcription factor TFIIA are generated from a single polypeptide in both mammalian and Drosophila cells (DeJong and Roeder, 1993; Ma et al., 1993; Yokomori et al., 1993). In addition, the herpes simplex virus VP16 accessory protein, host cell factor, which is necessary for activating transcription of the herpes simplex virus immediate-early genes, is a member of a family of proteins generated by the processing of a single 300 kDa precursor protein (Wilson et al., 1993). Another example is the virus-inducible processing of the IFN regulatory factor 2 protein (Palombella and Maniatis, 1992). In this case, IFN regulatory factor 2, which is a transcriptional repressor, is truncated and inactivated by inducers of IFN $\beta$  gene expression. A final example is provided by the membrane-bound sterol regulatory element-binding protein 1, which is processed in the absence of sterols to generate a transcriptionally active protein, while sterols repress transcription by inhibiting sterol regulatory element-binding protein 1 processing (Wang et al., 1994). At present, it is not known whether the proteasome is required for any of these examples of proteolytic processing of transcription factors.

The present finding of the activation of NF- $\kappa$ B by proteasomes demonstrates a new, unexpected role for the Ub-proteasome pathway in protein processing and degradation. In addition, this finding may also have medical applications. Because NF- $\kappa$ B plays an essential role in numerous immune and inflammatory responses, selective inhibition of proteasome function may provide a novel approach for blocking these processes in disease states.

#### Experimental Procedures

##### Materials

Ub, leupeptin, TPCK, apyrase, and the fluorogenic peptide succinyl-Leu-Leu-Val-Tyr-amidomethyl coumarin (Suc-LLVY-AMC) were all purchased from Sigma. Human TNF $\alpha$ , human IFN $\gamma$ , calpain inhibitor II, E64, AEBSF, and chymostatin were obtained from Boehringer-Mannheim. The proteasome inhibitors MG101, MG115, and MG132 were gifts from MyoGenics. Chymotrypsin inhibitors I and II were obtained from Calbiochem.  $^{125}$ I-lysozyme and Ub-conjugated  $^{125}$ I-lysozyme were gifts from O. Coux. Antibodies against the NF- $\kappa$ B subunit p50 were prepared as described in Thanos and Maniatis (1992). Antiserum (antibody 21) against I $\kappa$ B $\alpha$  (amino acids 5–20) was a gift from A. Baldwin.

##### Yeast Strains

The *S. cerevisiae* strains used in this work were gifts from S. Jentsch and W. Seufert. The yeast strains YW071 and YW074 (*MATa, his3Δ200, leu2-3, leu2-112, lys2-801, trp1-1(am), ura3-52*) encode a wild-type proteasome *PRE1* and a mutant *pre1-1* allele, respectively (Heinemeyer et al., 1991; Seufert and Jentsch, 1992).

##### Plasmids

Human p105 and a p60<sup>Th</sup> deletion construct were as described by Fan and Maniatis (1991). The p105 constructs were also subcloned into the yeast pADNS expression vector (Colicelli et al., 1989). Bacterial expression plasmids for histidine-tagged Ub and UbaR48 (from *Arabidopsis thaliana*) were from J. Callis (Beers and Callis, 1993). In the mutant, the lysine residue at position 48 has been altered by site-directed mutagenesis to encode an arginine.

#### Protein Extracts and Purification

HeLa cell cytoplasmic extract (S100) was described in Fan and Maniatis (1991). Pr<sup>-</sup> extracts were prepared by ultracentrifuging the S100 for 6 hr at 100,000  $\times$  g (Hegde et al., 1993), and the proteasome-enriched pellet fraction was resuspended in processing buffer (20 mM Tris [pH 7.5], 100 mM KCl). This fractionation was confirmed using  $^{125}$ I-lysozyme as a substrate in a degradation assay (Driscoll and Goldberg, 1990). The Pr<sup>-</sup> supernatant failed to degrade  $^{125}$ I-lysozyme unless it was recombined with proteasomes. In addition, proteasome activity in the original S100 and the pellet was demonstrated by hydrolysis of the fluorogenic substrate Suc-LLVY-AMC (Driscoll and Goldberg, 1990), while the Pr<sup>-</sup> supernatant lacked this proteasome activity. Reticulocyte fraction II (a gift from D. Hwang) was prepared as described in Driscoll and Goldberg (1990). Purified rabbit muscle 20S and 26S proteasomes were obtained from D. Hwang, and purified rabbit muscle 20S proteasomes were obtained from O. Coux. Highly purified 26S proteasomes were a gift from MyoGenics. Histidine-tagged Ub proteins were expressed in *E. coli* and were purified using Ni<sup>2+</sup>-NTA chromatography as described in Beers and Callis (1993).

#### In Vitro Processing Assay

The processing reaction was performed as described in Fan and Maniatis (1991), with a few modifications. In brief, the pcDNA1 p105 and p60Tth constructs were transcribed and translated in vitro with wheat germ extract (Promega) in the presence of [ $^{35}$ S]methionine (Amersham). The p105/p60Tth substrate proteins (4  $\mu$ l) in wheat germ extract were added to a 25  $\mu$ l processing reaction containing 12 mM Tris (pH 7.5), 60 mM KCl, 3.5 mM MgCl<sub>2</sub>, 1 mM ATP, and 20 mM creatine phosphate. HeLa cell extract (25–50  $\mu$ g) or fraction II was then added to the reaction mixtures for 1.0–1.5 hr at 30°C. Proteasomes, Ub, ATP $\gamma$ S (Boehringer-Mannheim), and various inhibitors were also added to the reactions where indicated. The reactions were then immunoprecipitated with anti-p50 antibody. The immunoprecipitated proteins were resolved by 7.5% SDS-PAGE.

#### Pulse-Chase and Western Blot Analysis

COS cells were transfected with either the pcDNA1 vector or pcDNA1 p105 using the DEAE-dextran method (Fan and Maniatis, 1991). After transfection (48 hr), the cells were treated with different inhibitors for 1 hr. Pulse-chase analysis was performed as described by Fan and Maniatis (1991). Cell lysates were immunoprecipitated with anti-p50 antibody, and the proteins were separated by 8% SDS-PAGE.

Yeast were transformed with the pADNS vector or pADNS p105. Transformants growing exponentially in selective medium were pulsed with 200  $\mu$ Ci of Tran<sup>35</sup>S-label (ICN Biomedicals) for 20 min and were chased with selective medium containing methionine and cysteine for different periods of time. Extracts were prepared by vortexing the yeast in RIPA buffer (Harlow and Lane, 1988) for 3 min in the presence of glass beads. Immunoprecipitation of the lysate with anti-p50 antibody and 10% SDS-PAGE analysis is described above.

Western blot analysis (10% SDS-PAGE) was performed according to Fan and Maniatis (1991).

#### Electrophoretic Mobility Shift Assay

Whole-cell extracts were prepared from untreated and cytokine-treated MG-63 and HeLa cells that had been pretreated with inhibitors for 1 hr. NF- $\kappa$ B binding activity was determined by an electrophoretic mobility shift assay, as described in Thanos and Maniatis (1992), using the PRDII probe from the IFN $\beta$  gene promoter. GAF DNA binding activity was measured using a –127 to –109 probe from the IFN regulatory factor 1 gene promoter (Sims et al., 1993).

#### Acknowledgments

We are grateful to C.-M. Fan for the p105 expression plasmids and for helpful discussions. We thank A. Baldwin for providing the anti-I $\kappa$ B $\alpha$  antibody, J. Callis for the histidine-tagged Ub constructs, P. Howley for the GST-Ub fusion construct, and O. Coux and D. Hwang for providing the  $^{125}$ I-lysozyme,  $^{125}$ I-Ub-lysozyme, fraction II, and the purified proteasomes. We are grateful to R. Stein, S. Brand, L. Dick, and other scientists at MyoGenics, Inc. for supplying the proteasome inhibitors, the purified 26S proteasomes, and their helpful advice. We thank S. Jentsch for the yeast strains used in this work and S. Liang and N.

Lehming for their helpful advice on the yeast expression experiments. We also thank M. L. Hedley and members of the Maniatis laboratory for materials, valuable discussions, and critical reading of the manuscript. This work was supported by the National Institutes of Health grants AI20642 to T. M. and GM46147 to A. L. G.

Received May 3, 1994; revised July 13, 1994.

#### References

Baeuerle, P. A. (1991). The inducible transcription activator NF- $\kappa$ B: regulation by distinct protein subunits. *Biochim. Biophys. Acta* 1072, 63–80.

Baeuerle, P. A., and Baltimore, D. (1988). I $\kappa$ B: a specific inhibitor of the NF- $\kappa$ B transcription factor. *Science* 242, 540–546.

Baeuerle, P. A., and Baltimore, D. (1989). A 65-kD subunit of active NF- $\kappa$ B is required for inhibition of NF- $\kappa$ B by I $\kappa$ B. *Genes Dev.* 3, 1689–1698.

Barr, P. J. (1991). Mammalian subtilisins: the long-sought dibasic processing endopeptidases. *Cell* 66, 1–3.

Beers, E. P., and Callis, J. (1993). Utility of polyhistidine-tagged ubiquitin in the purification of ubiquitin–protein conjugates and as an affinity ligand for the purification of ubiquitin-specific hydrolases. *J. Biol. Chem.* 268, 21645–21649.

Beg, A. A., and Baldwin, A. S., Jr. (1993). The I $\kappa$ B proteins: multifunctional regulators of rel/NF- $\kappa$ B transcription factors. *Genes Dev.* 7, 2064–2070.

Beg, A. A., Finco, T. S., Nantermet, P. V., and Baldwin, A. S., Jr. (1993). Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I $\kappa$ B $\alpha$ : a mechanism for NF- $\kappa$ B activation. *Mol. Cell. Biol.* 13, 3301–3310.

Blank, V., Kourilsky, P., and Israel, A. (1991). Cytoplasmic retention, DNA binding and processing of the NF- $\kappa$ B p50 precursor are controlled by a small region in its C-terminus. *EMBO J.* 10, 4159–4167.

Boes, B., Hengel, H., Ruppert, T., Multhaup, G., Koszinowski, U. H., and Kloetzel, P.-M. (1994). Interferon  $\gamma$  stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes. *J. Exp. Med.* 179, 901–909.

Bours, V., Villalobos, J., Burd, P. R., Kelly, K., and Siebenlist, U. (1990). Cloning of a mitogen-inducible gene encoding a  $\kappa$ B DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. *Nature* 348, 76–80.

Brown, K., Park, S., Kanno, T., Franzoso, G., and Siebenlist, U. (1993). Mutual regulation of the transcriptional activator NF- $\kappa$ B and its inhibitor, I $\kappa$ B $\alpha$ . *Proc. Natl. Acad. Sci. USA* 90, 2532–2536.

Capobianco, A. J., Chang, D., Mosialos, G., and Gilmore, T. D. (1992). p105, the NF- $\kappa$ B p50 precursor protein, is one of the cellular proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. *J. Virol.* 66, 3758–3767.

Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* 243, 1576–1583.

Colicelli, J., Birchmeier, C., Michaeli, T., O'Neil, K., Riggs, M., and Wigler, M. (1989). Isolation and characterization of a mammalian gene encoding a high-affinity cAMP phosphodiesterase. *Proc. Natl. Acad. Sci. USA* 86, 3599–3603.

Collins, T. (1993). Endothelial nuclear factor- $\kappa$ B and the initiation of the atherosclerotic lesion. *Lab. Invest.* 68, 499–508.

DeJong, J., and Roeder, R. G. (1993). A single cDNA, hTFIIB $\alpha$ , encodes both the p35 and p19 subunits of human TFIIIB. *Genes Dev.* 7, 2220–2234.

Driscoll, J., and Goldberg, A. L. (1990). The proteasome (multicatalytic protease) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins. *J. Biol. Chem.* 265, 4789–4792.

Eytan, E., Ganot, D., Armon, T., and Hershko, A. (1989). ATP-dependent incorporation of 20S protease into the 26S complex that

degrades proteins conjugated to ubiquitin. *Proc. Natl. Acad. Sci. USA* 86, 7751–7755.

Fan, C.-M., and Maniatis, T. (1991). Generation of p50 subunit of NF- $\kappa$ B by processing of p105 through an ATP-dependent pathway. *Nature* 354, 395–398.

Frantz, B., Nordby, E. C., Bren, G., Steffan, N., Paya, C. V., Kincaid, R. L., Tocci, M. J., O'Keefe, S. J., and O'Neill, E. A. (1994). Calcineurin acts in synergy with PMA to inactivate I $\kappa$ B/MAD3, an inhibitor of NF- $\kappa$ B. *EMBO J.* 13, 861–870.

Gaczynska, M., Rock, K. L., and Goldberg, A. L. (1993).  $\gamma$ -interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* 365, 264–267.

Geisler, R., Bergmann, A., Hiromi, Y., and Nüsslein-Volhard, C. (1992). *Cactus*, a gene involved in dorsoventral pattern formation of Drosophila, is related to the I $\kappa$ B gene family of vertebrates. *Cell* 71, 613–621.

Ghosh, S., and Baltimore, D. (1990). Activation *in vitro* of NF- $\kappa$ B by phosphorylation of its inhibitor I $\kappa$ B. *Nature* 344, 678–682.

Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P., and Baltimore, D. (1990). Cloning of the p50 DNA binding subunit of NF- $\kappa$ B: homology to *rel* and *dorsal*. *Cell* 62, 1019–1029.

Goldberg, A. L. (1992). The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. *Eur. J. Biochem.* 203, 9–23.

Goldberg, A. L., and Rock, K. L. (1992). Proteolysis, proteasomes and antigen presentation. *Nature* 357, 375–379.

Grilli, M., Chiu, J. J.-S., and Lenardo, M. J. (1993). NF- $\kappa$ B and Rel: participants in a multiform transcriptional regulatory system. *Int. Rev. Cytol.* 143, 1–62.

Hartow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Hegde, A. N., Goldberg, A. L., and Schwartz, J. H. (1993). Regulatory subunits of cAMP-dependent protein kinases are degraded after conjugation to ubiquitin: a molecular mechanism underlying long-term synaptic plasticity. *Proc. Natl. Acad. Sci. USA* 90, 7436–7440.

Heinemeyer, W., Kleinschmidt, J. A., Saidowski, J., Escher, C., and Wolf, D. H. (1991). Proteinase ycsE, the yeast proteasome/multicatalytic proteinase: mutants unravel its function in stress-induced proteolysis and uncover its necessity for cell survival. *EMBO J.* 10, 555–562.

Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y., and Baeuerle, P. A. (1993). Rapid proteolysis of I $\kappa$ B- $\alpha$  is necessary in the activation of transcription factor NF- $\kappa$ B. *Nature* 365, 182–185.

Hershko, A., and Ciechanover, A. (1992). The ubiquitin system for protein degradation. *Annu. Rev. Biochem.* 61, 761–807.

Hough, R., Pratt, G., and Rechsteiner, M. (1987). Purification of two high molecular weight proteases from rabbit reticulocyte lysates. *J. Biol. Chem.* 262, 8303–8313.

Inoue, J., Kerr, L. D., Kakizuka, A., and Verma, I. M. (1992). I $\kappa$ B $\gamma$ , a 70 kd protein identical to the C-terminal half of p110 NF- $\kappa$ B: a new member of the I $\kappa$ B family. *Cell* 68, 1109–1120.

Jentsch, S. (1992). The ubiquitin-conjugation system. *Annu. Rev. Genet.* 23, 179–207.

Kidd, S. (1992). Characterization of the Drosophila cactus locus and analysis of interactions between cactus and dorsal proteins. *Cell* 71, 623–635.

Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., LeBail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A., and Israel, A. (1990). The DNA binding subunit of NF- $\kappa$ B is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell* 62, 1007–1018.

Liou, H.-C., and Baltimore, D. (1993). Regulation of the NF- $\kappa$ B/rel transcription factor and I $\kappa$ B inhibitor system. *Curr. Opin. Cell Biol.* 5, 477–487.

Liou, H.-C., Nolan, G. P., Ghosh, S., Fujita, T., and Baltimore, D. (1992). The NF- $\kappa$ B precursor, p105, contains an internal I $\kappa$ B-like inhibitor that preferentially inhibits p50. *EMBO J.* 11, 3003–3009.

Ma, D., Watanabe, H., Mermelstein, F., Admon, A., Oguri, K., Sun, X., Wada, T., Imai, T., Shiroya, T., Reinberg, D., and Handa, H. (1993). Isolation of a cDNA encoding the largest subunit of TFIIA reveals functions important for activated transcription. *Genes Dev.* 7, 2246–2257.

Matthews, W., Tanaka, K., Driscoll, J., Ichihara, A., and Goldberg, A. L. (1989). Involvement of the proteasome in various degradative processes in mammalian cells. *Proc. Natl. Acad. Sci. USA* 86, 2597–2601.

Mellits, K. H., Hay, R. T., and Goodbourn, S. (1993). Proteolytic degradation of MAD3 (I $\kappa$ B $\alpha$ ) and enhanced processing of the NF- $\kappa$ B precursor p105 are obligatory steps in the activation of NF- $\kappa$ B. *Nucl. Acids Res.* 21, 5059–5066.

Mercuro, F., DiDonato, J. A., Rosette, C., and Karin, M. (1993). p105 and p98 precursor proteins play an active role in NF- $\kappa$ B-mediated signal transduction. *Genes Dev.* 7, 705–718.

Michalek, M. T., Grant, E. P., Gramm, C., Goldberg, A. L., and Rock, K. L. (1993). A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature* 363, 552–554.

Nabel, G., and Baltimore, D. (1987). An inducible factor activates expression of human immunodeficiency virus in T cells. *Nature* 326, 711–713.

Neumann, M., Tsapos, K., Scheppeler, J. A., Ross, J., and Franz, B. R., Jr. (1992). Identification of complex formation between two intracellular tyrosine kinase substrates: human c-Rel and the p105 precursor of p50 NF- $\kappa$ B. *Oncogene* 7, 2095–2104.

Nolan, G. P., Ghosh, S., Lion, H.-C., Tempst, P., and Baltimore, D. (1991). DNA binding and I $\kappa$ B inhibition of the cloned p65 subunit of NF- $\kappa$ B, a *rel*-related polypeptide. *Cell* 64, 961–969.

Palombella, V. J., and Maniatis, T. (1992). Inducible processing of interferon regulatory factor-2. *Mol. Cell. Biol.* 12, 3325–3336.

Rechsteiner, M., Hoffman, L., and Dubiel, W. (1993). The multicatalytic and 26S proteases. *J. Biol. Chem.* 268, 6065–6068.

Rice, N. R., MacKichan, M. L., and Israel, A. (1992). The precursor of NF- $\kappa$ B p50 has I $\kappa$ B-like functions. *Cell* 71, 243–253.

Riviere, Y., Blank, V., Kourilsky, P., and Israel, A. (1991). Processing of the precursor of NF- $\kappa$ B by the HIV-1 protease during acute infection. *Nature* 350, 625–626.

Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994). Inhibitors of the proteasome block degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78, this issue.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234, 384–388.

Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C.-H., Maher, M., Baeuerle, P. A., and Rosen, C. A. (1991). Isolation of a *rel*-related human cDNA that potentially encodes the 65-kD subunit of NF- $\kappa$ B. *Science* 251, 1490–1493.

Scheffner, M., Munger, K., Huibregtsse, J. M., and Howley, P. M. (1992). Targeted degradation of the retinoblastoma protein by human papillomavirus E7-E6 fusion proteins. *EMBO J.* 11, 2425–2431.

Scheffner, M., Huibregtsse, J. M., Vierstra, R. D., and Howley, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75, 495–505.

Seufert, W., and Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *EMBO J.* 9, 543–550.

Seufert, W., and Jentsch, S. (1992). *In vivo* function of the proteasome in the ubiquitin pathway. *EMBO J.* 11, 3077–3080.

Shelton, C. A., and Wasserman, S. A. (1993). *palle* encodes a protein kinase required to establish dorsoventral polarity in the Drosophila embryo. *Cell* 72, 515–525.

Shirakawa, F., and Mizel, S. B. (1989). *In vitro* activation and nuclear translocation of NF- $\kappa$ B catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Mol. Cell. Biol.* 9, 2424–2430.

Sims, S. H., Cha, Y., Romine, M. F., Gao, P. Q., Gottlieb, K., and Deisseroth, A. B. (1993). A novel interferon-inducible domain: structural and functional analysis of the human interferon regulatory factor 1 gene promoter. *Mol. Cell. Biol.* 13, 690–702.

Sun, S.-C., Ganchi, P. A., Ballard, D. W., and Greene, W. C. (1993). NF- $\kappa$ B controls expression of inhibitor I $\kappa$ B $\alpha$ : evidence for an inducible autoregulatory pathway. *Science* 259, 1912–1915.

Sun, S.-C., Ganchi, P. A., Beraud, C., Ballard, D. W., and Greene, W. C. (1994). Autoregulation of the NF- $\kappa$ B transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. Proc. Natl. Acad. Sci. USA 91, 1346–1350.

Tanaka, K., Tamura, T., Yoshimura, T., and Ichihara, A. (1992). Proteasomes: protein and gene structures. New Biol. 4, 173–187.

Thanos, D., and Maniatis, T. (1992). The high mobility group protein HMG I(Y) is required for NF- $\kappa$ B-dependent virus induction of the human IFN- $\beta$  gene. Cell 71, 777–789.

Varshavsky, A. (1992). The N-end rule. Cell 69, 725–735.

Visnitsky, A., Michaud, C., Powers, J. J., and Orlowski, M. (1992). Inhibition of the chymotrypsin-like activity of the pituitary multicatalytic proteasome complex. Biochemistry 31, 9421–9428.

Wang, X., Sato, R., Brown, M. S., Hua, X., and Goldstein, J. L. (1994). SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell 77, 53–62.

Whalen, A. M., and Steward, R. (1993). Dissociation of the dorsal-cactus complex and phosphorylation of the dorsal protein correlate with the nuclear localization of dorsal. J. Cell Biol. 123, 523–534.

Wilson, A. C., LeMarco, K., Peterson, M. G., and Herr, W. (1993). The VP16 accessory protein HCF is a family of polypeptides processed from a large precursor protein. Cell 74, 115–125.

Yokomori, K., Admon, A., Goodrich, J. A., Chen, J.-L., and Tjian, R. (1993). *Drosophila* TFIIA-L is processed into two subunits that are associated with the TBP/TAF complex. Genes Dev. 7, 2235–2245.

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